Certificate of Mailing

Date of Deposit: June 20, 2003 Label Number: EL 956997873 US

I hereby certify under 37 C.F.R. § 1.10 that this correspondence is being deposited with the United States Postal Service as **"Express Mail Post Office to Addressee"** with sufficient postage on the date indicated above and is addressed to Mail Stop Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Amy Beardsley
Printed name of person mailing correspondence

Signature of person mailing correspondence

APPLICATION

FOR

UNITED STATES LETTERS PATENT

APPLICANTS: ROBERT G. KORNELUK, ALEXANDER E. MACKENZIE, STEPHEN BAIRD, and PETER LISTON

TITLE: MAMMALIAN IAP GENE FAMILY, PRIMERS, PROBES AND DETECTION METHODS

MAMMALIAN IAP GENE FAMILY, PRIMERS, PROBES AND DETECTION METHODS

Cross Reference to Related Applications

This application is a continuation of U.S.S.N. 09/011,356, filed February 4, 1998 (now pending), which is a U.S. National Phase application of PCT/IB/96/01022, filed August 5, 1996, and published in English under PCT article 21(2), which claims benefit from U.S.S.N. 08/576,956, filed December 22, 1995 (now U.S. Patent No. 6,156,535), which is a continuation-in-part of U.S.S.N. 08/511,485, filed August 4, 1995 (now U.S. Patent No. 5,919,912), all of which are hereby incorporated by reference in their entirety.

Background of the Invention

The invention relates to apoptosis.

There are two general ways by which cells die. The most easily recognized way is by necrosis, which is usually caused by an injury that is severe enough to disrupt cellular homeostasis. Typically, the cell's osmotic pressure is disturbed and, consequently, the cell swells and then ruptures. When the cellular contents are spilled into the surrounding tissue space, an inflammatory response often ensues.

The second general way by which cells die is referred to as apoptosis, or programmed cell death. Apoptosis often occurs so rapidly that it is difficult to detect. This may help to explain why the involvement of apoptosis in a wide spectrum of biological processes has only recently been recognized.

The apoptosis pathway has been highly conserved throughout evolution, and plays a critical role in embryonic development, viral pathogenesis, cancer, autoimmune disorders, and neurodegenerative disease. For example, inappropriate apoptosis may cause or contribute to AIDS, Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS), retinitis pigmentosa and other diseases of the retina, myelodysplastic syndrome (e.g. aplastic anemia), toxin-induced liver disease, including alcoholism, and ischemic injury (e.g. myocardial infarction, stroke, and reperfusion injury). Conversely, the failure of an apoptotic response has been implicated in the development of cancer, particularly follicular lymphoma, p53-mediated carcinomas, and hormone-dependent tumors, in autoimmune disorders, such as lupus erythematosis and multiple sclerosis, and in viral infections, including those associated with herpes virus, poxvirus, and adenovirus.

In patients infected with HIV-1, mature $CD4^+$ T lymphocytes respond to stimulation from mitogens or super-antigens by undergoing apoptosis. However, the great majority of these cells are not infected with the virus. Thus, inappropriate antigen-induced apoptosis could be responsible for the destruction of this vital part of the immune system in the early stages of HIV infection.

Baculoviruses encode proteins that are termed inhibitors of apoptosis proteins (IAPs) because they inhibit the apoptosis that would otherwise occur when insect cells are infected by the virus. These proteins are thought to work in a manner that is independent of other viral proteins. The baculovirus IAP genes include sequences encoding a ring zinc finger-like motif (RZF), which is presumed to be directly involved in DNA binding, and two N-terminal domains that consist of a 70 amino acid repeat motif termed a BIR domain (Baculovirus IAP Repeat).

Summary of the Invention

In general, the invention features a substantially pure DNA molecule, such as a genomic, cDNA, or synthetic DNA molecule, that encodes a mammalian IAP polypeptide. This DNA may be incorporated into a vector, into a cell, which may be a mammalian, yeast, or bacterial cell, or into a transgenic animal or embryo thereof. In preferred embodiments, the DNA molecule is a murine gene (e.g., *m-xiap*, *m-hiap-1*, or *m-hiap-2*) or a human gene (e.g., *xiap*, *hiap-1*, or *hiap-2*). In most preferred embodiments the IAP gene is a human IAP gene. In other various preferred embodiments, the cell is a transformed cell. In related aspects, the invention features a transgenic animal containing a transgene that encodes an IAP polypeptide that is expressed in or delivered to tissue normally susceptible to apoptosis, i.e., to a tissue that may be harmed by either the induction or repression of apoptosis. In yet another aspect, the invention features DNA encoding fragments of IAP polypeptides including the BIR domains and the RZF domains provided herein.

In specific embodiments, the invention features DNA sequences substantially identical to the DNA sequences shown in Figs. 1-6, or fragments thereof. In another aspect, the invention also features RNA which is encoded by the DNA described herein. Preferably, the RNA is mRNA. In another embodiment the RNA is antisense RNA.

In another aspect, the invention features a substantially pure polypeptide having a sequence substantially identical to one of the IAP amino acid sequences shown in Figs. 1-6.

In a second aspect, the invention features a substantially pure DNA which includes a promoter capable of expressing the IAP gene in a cell susceptible to apoptosis. In preferred embodiments, the IAP gene is *xiap*, *hiap-1*, or *hiap-2*.

Most preferably, the genes are human or mouse genes. The gene encoding HIAP-2 may be the full-length gene, as shown in Fig. 3, or a truncated variant, such as a variant having a deletion of the sequence boxed in Fig. 3.

In preferred embodiments, the promoter is the promoter native to an IAP gene. Additionally, transcriptional and translational regulatory regions are, preferably, those native to an IAP gene. In another aspect, the invention provides transgenic cell lines and transgenic animals. The transgenic cells of the invention are preferably cells that are altered in their apoptotic response. In preferred embodiments, the transgenic cell is a fibroblast, neuronal cell, a lymphocyte cell, a glial cell, an embryonic stem cell, or an insect cell. Most preferably, the neuron is a motor neuron and the lymphocyte is a $CD4^+$ T cell.

In another aspect, the invention features a method of inhibiting apoptosis that involves producing a transgenic cell having a transgene encoding an IAP polypeptide. The transgene is integrated into the genome of the cell in a way that allows for expression. Furthermore, the level of expression in the cell is sufficient to inhibit apoptosis.

In a related aspect, the invention features a transgenic animal, preferably a mammal, more preferably a rodent, and most preferably a mouse, having either increased copies of at least one IAP gene inserted into the genome (mutant or wild-type), or a knockout of at least one IAP gene in the genome. The transgenic animals will express either an increased or a decreased amount of IAP polypeptide, depending on the construct used and the nature of the genomic alteration. For example, utilizing a nucleic acid molecule that encodes all or part of an IAP to engineer a knockout mutation in an IAP gene would generate an animal with decreased expression of either all or part of the corresponding IAP polypeptide. In contrast, inserting exogenous copies of all or part of an IAP gene

into the genome, preferably under the control of active regulatory and promoter elements, would lead to increased expression or the corresponding IAP polypeptide.

In another aspect, the invention features a method of detecting an IAP gene in a cell by contacting the IAP gene, or a portion thereof (which is greater than 9 nucleotides, and preferably greater than 18 nucleotides in length), with a preparation of genomic DNA from the cell. The IAP gene and the genomic DNA are brought into contact under conditions that allow for hybridization (and therefore, detection) of DNA sequences in the cell that are at least 50% identical to the DNA encoding HIAP-1, HIAP-2, or XIAP polypeptides.

In another aspect, the invention features a method of producing an IAP polypeptide. This method involves providing a cell with DNA encoding all or part of an IAP polypeptide (which is positioned for expression in the cell), culturing the cell under conditions that allow for expression of the DNA, and isolating the IAP polypeptide. In preferred embodiments, the IAP polypeptide is expressed by DNA that is under the control of a constitutive or inducible promotor. As described herein, the promotor may be a heterologous promotor.

In another aspect, the invention features substantially pure mammalian IAP polypeptide. Preferably, the polypeptide includes an amino acid sequence that is substantially identical to all, or to a fragment of, the amino acid sequence shown in any one of Figs. 1-4. Most preferably, the polypeptide is the XIAP, HIAP-1, HIAP-2, M-XIAP, M-HIAP-1, or M-HIAP-2 polypeptide. Fragments including one or more BIR domains (to the exclusion of the RZF), the RZF domain (to the exclusion of the BIR domains), and a RZF domain with at least one BIR domain, as provided herein, are also a part of the invention.

In another aspect, the invention features a recombinant mammalian polypeptide that is capable of modulating apoptosis. The polypeptide may include at least a RZF domain and a BIR domain as defined herein. In preferred embodiments, the invention features (a) a substantially pure polypeptide, and (b) an oligonucleotide encoding the polypeptide. In instances were the polypeptide includes a RZF domain, the RZF domain will have a sequence conforming to: Glu-Xaal-Xaal-Xaal- Xaal-Xaal-Xaal-Xaa2-Xaal-Xaal-Xaal-Cys-Lys-Xaa3-Cys-Met-Xaal-Xaal-Xaal-Xaal-Xaal-Xaa3-Xaal-Phe-Xaal-Pro-Cys-Gly-His-Xaal-Xaal-Xaal-Cys-Xaal-Xaal-Cys-Ala-Xaal-Xaal-Xaal-Xaal-Xaal-Cys-Pro-Xaal-Cys, where Xaal is any amino acid, Xaa2 is Glu or Asp, Xaa3 is Val or Ile (SEQ ID NO: l); and where the polypeptide includes at least one BIR domain, the BIR domain will have a sequence conforming to: Xaal-Xaal-Xaal-Arg-Leu-Xaal-Thr-Phe-Xaal-Xaal-Trp-Pro-Xaa2-Xaal-Xaal-Xaa2-Xaa2-Xaal-Xaal-Xaal-Xaal-Leu-Ala-Xaal-Ala-Gly-Phe-Tyr-Tyr-Xaal-Gly-Xaal-Xaal-Asp-Xaal-Val-Xaal-Cys-Phe-Xaal-Cys-Xaal-Xaal-Xaal-Xaal-Xaal-Xaal-Trp-Xaal-Xaal-Xaal-Asp-Xaal-Xaal-Xaal-Xaal-Xaal-His-Xaal-Xaal-Xaal-Xaal-Pro-Xaal-Cys-Xaal-Phe-Val, where Xaal may be any amino acid and Xaa2 may be any amino acid or may be absent (SEQ ID NO: 2).

In various preferred embodiments the polypeptide has at least two or, more preferably at least three BIR domains, the RZF domain has one of the IAP sequences shown in Fig. 6, and the BIR domains are comprised of BIR domains shown in Fig. 5. In other preferred embodiments the BIR domains are at the amino terminal end of the protein relative to the RZF domain, which is at or near the carboxyl terminus of the polypeptide.

In another aspect, the invention features an IAP gene isolated according to the method involving: (a) providing a sample of DNA; (b) providing a pair of

oligonucleotides having sequence homology to a conserved region of an IAP disease-resistance gene; (c) combining the pair of oligonucleotides with the cell DNA sample under conditions suitable for polymerase chain reaction-mediated DNA amplification; and (d) isolating the amplified IAP gene or fragment thereof.

In preferred embodiments, the amplification is carried out using a reverse-transcription polymerase chain reaction, for example, the RACE method. In another aspect, the invention features an IAP gene isolated according to the method involving: (a) providing a preparation of DNA; (b) providing a detectably labelled DNA sequence having homology to a conserved region of an IAP gene; (c) contacting the preparation of DNA with the detectably-labelled DNA sequence under hybridization conditions providing detection of genes having 50% or greater nucleotide sequence identity; and (d) identifying an IAP gene by its association with the detectable label.

In another aspect, the invention features an IAP gene isolated according to the method involving: (a) providing a cell sample; (b) introducing by transformation into the cell sample a candidate IAP gene; (c) expressing the candidate IAP gene within the cell sample; and (d) determining whether the cell sample exhibits an altered apoptotic response, whereby a response identifies an IAP gene.

In another aspect, the invention features a method of identifying an IAP gene in a cell, involving: (a) providing a preparation of cellular DNA (for example, from the human genome or a cDNA library (such as a cDNA library isolated from a cell type which undergoes apoptosis); (b) providing a detectably-labelled DNA sequence (for example, prepared by the methods of the invention) having homology to a conserved region of an IAP gene; (c) contacting the preparation of cellular DNA with the detectably-labelled DNA sequence under

hybridization conditions providing detection of genes having 50% nucleotide or greater sequence identity; and (d) identifying an IAP gene by its association with the detectable label.

In another aspect, the invention features a method of isolating an IAP gene from a recombinant library, involving: (a) providing a recombinant library; (b) contacting the library with a detectably-labelled gene fragment produced according to the PCR method of the invention under hybridization conditions providing detection of genes having 50% or greater nucleotide sequence identity; and (c) isolating an IAP gene by its association with the detectable label. In another aspect, the invention features a method of identifying an IAP gene involving: (a) providing a cell tissue sample; (b) introducing by transformation into the cell sample a candidate IAP gene; (c) expressing the candidate IAP gene within the cell sample; and (d) determining whether the cell sample exhibits inhibition of apoptosis, whereby a change in (i.e. modulation of) apoptosis identifies an IAP gene. Preferably, the cell sample is a cell type that may be assayed for apoptosis (e.g., T cells, B cells, neuronal cells, baculovirus-infected insect cells, glial cells, embryonic stem cells, and fibroblasts). The candidate IAP gene is obtained, for example, from a cDNA expression library, and the response assayed is the inhibition of apoptosis.

In another aspect, the invention features a method of inhibiting apoptosis in a mammal wherein the method includes: (a) providing DNA encoding at least one IAP polypeptide to a cell that is susceptible to apoptosis; wherein the DNA is integrated into the genome of the cell and is positioned for expression in the cell; and the IAP gene is under the control of regulatory sequences suitable for controlled expression of the gene(s); wherein the IAP transgene is expressed at a level sufficient to inhibit apoptosis relative to a cell

lacking the IAP transgene. The DNA integrated into the genome may encode all or part of an IAP polypeptide. It may, for example, encode a ring zinc finger and one or more BIR domains. In contrast, it may encode either the ring zinc finger alone, or one or more BIR domains alone. Skilled artisans will appreciate that IAP polypeptides may also be administered directly to inhibit undesirable apoptosis.

In a related aspect, the invention features a method of inhibiting apoptosis by producing a cell that has integrated, into its genome, a transgene that includes the IAP gene, or a fragment thereof. The IAP gene may be placed under the control of a promoter providing constitutive expression of the IAP gene. Alternatively, the IAP transgene may be placed under the control of a promoter that allows expression of the gene to be regulated by environmental stimuli. For example, the IAP gene may be expressed using a tissue-specific or cell type-specific promoter, or by a promoter that is activated by the introduction of an external signal or agent, such as a chemical signal or agent. In preferred embodiments the cell is a lymphocyte, a neuronal cell, a glial cell, or a fibroblast. In other embodiments, the cell in an HIV-infected human, or in a mammal suffering from a neurodegenerative disease, an ischemic injury, a toxin-induced liver disease, or a myelodysplastic syndrome.

In a related aspect, the invention provides a method of inhibiting apoptosis in a mammal by providing an apoptosis-inhibiting amount of IAP polypeptide. The IAP polypeptide may be a full-length polypeptide, or it may be one of the fragments described herein.

In another aspect, the invention features a purified antibody that binds specifically to an IAP family protein. Such an antibody may be used in any standard immunodetection method for the identification of an IAP polypeptide. Preferably, the antibody binds specifically to XIAP, HIAP-1, or HIAP-2. In

various embodiments, the antibody may react with other IAP polypeptides or may be specific for one or a few IAP polypeptides. The antibody may be a monoclonal or a polyclonal antibody. Preferably, the antibody reacts specifically with only one of the IAP polypeptides, for example, reacts with murine and human XIAP, but not with HIAP-1 or HIAP-2 from other mammalian species.

The antibodies of the invention may be prepared by a variety of methods. For example, the IAP polypeptide, or antigenic fragments thereof, can be administered to an animal in order to induce the production of polyclonal antibodies. Alternatively, antibodies used as described herein may be monoclonal antibodies, which are prepared using hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In *Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, NY, 1981). The invention features antibodies that specifically bind human or murine IAP polypeptides, or fragments thereof. In particular the invention features "neutralizing" antibodies. By "neutralizing" antibodies is meant antibodies that interfere with any of the biological activities of IAP polypeptides, particularly the ability of IAPs to inhibit apoptosis. The neutralizing antibody may reduce the ability of IAP polypeptides to inhibit polypeptides by, preferably 50%, more preferably by 70, and most preferably by 90% or more. Any standard assay of apoptosis, including those described herein, may be used to assess neutralizing antibodies.

In addition to intact monoclonal and polyclonal anti-IAP antibodies, the invention features various genetically engineered antibodies, humanized antibodies, and antibody fragments, including F(ab')2, Fab', Fab, Fv and sFv fragments. Antibodies can be humanized by methods known in the art, e.g., monoclonal antibodies with a desired binding specificity can be commercially

humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA). Fully human antibodies, such as those expressed in transgenic animals, are also features of the invention (Green et al., Nature Genetics 7:13, 1994).

Ladner (U.S. Patent 4,946,778 and 4,704,692) describes methods for preparing single polypeptide chain antibodies. Ward et al. (Nature 341:544, 1989) describe the preparation of heavy chain variable domains, which they term "single domain antibodies," which have high antigen-binding affinities. McCafferty et al. (Nature 348:552, 1990) show that complete antibody V domains can be displayed on the surface of fd bacteriophage, that the phage bind specifically to antigen, and that rare phage (one in a million) can be isolated after affinity chromatography. Boss et al. (U.S. Patent 4,816,397) describe various methods for producing immunoglobulins, and immunologically functional fragments thereof, which include at least the variable domains of the heavy and light chain in a single host cell. Cabilly et al. (U.S. Patent 4,816,567) describe methods for preparing chimeric antibodies.

In another aspect, the invention features a method of identifying a compound that modulates apoptosis. The method includes providing a cell expressing an IAP polypeptide, contacting the cell with a candidate compound, and monitoring the expression of an IAP gene. An alteration in the level of expression of the IAP gene indicates the presence of a compound which modulates apoptosis. The compound may be an inhibitor or an enhancer of apoptosis. In various preferred embodiments, the cell is a fibroblast, a neuronal cell, a glial cell, a lymphocyte (T cell or B cell), or an insect cell; the polypeptide expression being monitored is XIAP, HIAP-1, HIAP-2, M-XIAP, M-HIAP-1, or M-HIAP-2 (i.e., human or murine).

In a related aspect, the invention features methods of detecting compounds that modulate apoptosis using the interaction trap technology and IAP polypeptides, or fragments thereof, as a component of the bait. In preferred embodiments, the compound being tested as a modulator of apoptosis is also a polypeptide.

In another aspect, the invention features a method for diagnosing a cell proliferation disease, or an increased likelihood of such a disease, using an IAP nucleic acid probe or antibody. Preferably, the disease is a cancer. Most preferably, the disease is selected from the group consisting of promyelocytic leukemia, a HeLa-type carcinoma, chronic myelogenous leukemia (preferably using *xiap* or *hiap-2* related probes), lymphoblastic leukemia (preferably using a *xiap* related probe), Burkitt's lymphoma (preferably using an *hiap-1* related probe), colorectal adenocarcinoma, lung carcinoma, and melanoma (preferably using a *xiap* probe). Preferably, a diagnosis is indicated by a 2-fold increase in expression or activity, more preferably, at least a 10-fold increase in expression or activity.

Skilled artisans will recognize that a mammalian IAP, or a fragment thereof (as described herein), may serve as an active ingredient in a therapeutic composition. This composition, depending on the IAP or fragment included, may be used to modulate apoptosis and thereby treat any condition that is caused by a disturbance in apoptosis.

In addition, apoptosis may be induced in a cell by administering to the cell a negative regulator of the IAP-dependent anti-apoptotic pathway. The negative regulator may be, but is not limited to, an IAP polypeptide that includes a ring zinc finger, and an IAP polypeptide that includes a ring zinc finger and lacks at least one BIR domain. Alternatively, apoptosis may be induced in the cell by

administering a gene encoding an IAP polypeptide, such as these two polypeptides. In yet another method, the negative regulator may be a purified antibody, or a fragment thereof, that binds specifically to an IAP polypeptide. For example, the antibody may bind to an approximately 26 kDa cleavage product of an IAP polypeptide that includes at least one BIR domain but lacks a ring zinc finger domain. The negative regulator may also be an IAP antisense mRNA molecule.

As summarized above, an IAP nucleic acid, or an IAP polypeptide may be used to modulate apoptosis. Furthermore, an IAP nucleic acid, or an IAP polypeptide, may be used in the manufacture of a medicament for the modulation of apoptosis.

By "IAP gene" is meant a gene encoding a polypeptide having at least one BIR domain and a ring zinc finger domain which is capable of modulating (inhibiting or enhancing) apoptosis in a cell or tissue when provided by other intracellular or extracellular delivery methods. In preferred embodiments the IAP gene is a gene having about 50% or greater nucleotide sequence identity to at least one of the IAP amino acid encoding sequences of Figs. 1-4 or portions thereof. Preferably, the region of sequence over which identity is measured is a region encoding at least one BIR domain and a ring zinc finger domain. Mammalian IAP genes include nucleotide sequences isolated from any mammalian source. Preferably, the mammal is a human.

The term "IAP gene" is meant to encompass any member of the family of apoptosis inhibitory genes, which are characterized by their ability to modulate apoptosis. An IAP gene may encode a polypeptide that has at least 20%, preferably at least 30%, and most preferably at least 50% amino acid sequence identity with at least one of the conserved regions of one of the IAP members

described herein (i.e., either the BIR or ring zinc finger domains from the human or murine *xiap*, *hiap-1* and *hiap-2*). Representative members of the IAP gene family include, without limitation, the human and murine *xiap*, *hiap-1*, and *hiap-2* genes.

By "IAP protein" or "IAP polypeptide" is meant a polypeptide, or fragment thereof, encoded by an IAP gene.

By "BIR domain" is meant a domain having the amino acid sequence of the consensus sequence: Xaal-Xaal-Xaal-Arg-Leu-Xaal-Thr-Phe-Xaal-Xaal-Trp-Pro-Xaa2-Xaal-Xaal-Xaa2-Xaa2-Xaal-Xaal-Xaal-Xaal-Leu-Ala-Xaal-Ala-Gly-Phe-Tyr-Tyr-Xaal-Gly-Xaal-Xaal-Asp-Xaal-Val-Xaal-Cys-Phe-Xaal-Cys-Xaal-Xaal- Xaal-Xaal-Xaal-Xaal-Trp-Xaal-Xaal-Xaal-Asp-Xaal-Xaal-Xaal- Xaal-Xaal-His-Xaal-Xaal-Xaal-Xaal-Pro-Xaal-Cys-Xaal-Phe-Val, wherein Xaal is any amino acid and Xaa2 is any amino acid or is absent (SEQ ID NO: 2). Preferably, the sequence is substantially identical to one of the BIR domain sequences provided herein for XIAP, HIAP-1, or HIAP-2.

By "ring zinc finger" or "RZF" is meant a domain having the amino acid sequence of the consensus sequence: Glu-Xaal-Xaal-Xaal-Xaal-Xaal-Xaal-Xaa2-Xaal-Xaal-Xaal-Cys- Lys-Xaa3-Cys-Met-Xaal-Xaal-Xaal-Xaal-Xaal-Xaa3-Xaal-Phe-Xaal-Pro-Cys-Gly-His-Xaal-Xaal-Xaal-Cys-Xaal-Xaal-Cys-Ala- Xaal-Xaal-Xaal-Xaal-Xaal-Cys-Pro-Xaal-Cys, wherein Xaal is any amino acid, Xaa2 is Glu or Asp, and Xaa3 is Val or Ile (SEQ ID NO: 1).

Preferably, the sequence is substantially identical to the RZF domains provided herein for the human or murine XIAP, HIAP-1, or HIAP-2.

By "modulating apoptosis" or "altering apoptosis" is meant increasing or decreasing the number of cells that would otherwise undergo apoptosis in a given cell population. Preferably, the cell population is selected

from a group including T cells, neuronal cells, fibroblasts, or any other cell line known to undergo apoptosis in a laboratory setting (e.g., the baculovirus infected insect cells). It will be appreciated that the degree of modulation provided by an IAP or modulating compound in a given assay will vary, but that one skilled in the art can determine the statistically significant change in the level of apoptosis which identifies an IAP or a compound which modulates an IAP.

By "inhibiting apoptosis" is meant any decrease in the number of cells which undergo apoptosis relative to an untreated control. Preferably, the decrease is at least 25%, more preferably the decrease is 50%, and most preferably the decrease is at least one-fold.

By "polypeptide" is meant any chain of more than two amino acids, regardless of post-translational modification such as glycosylation or phosphorylation.

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably 85%, more preferably 90%, and most preferably 95% homology to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This

software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "substantially pure polypeptide" is meant a polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is an IAP polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure IAP polypeptide may be obtained, for example, by extraction from a natural source (e.g. a fibroblast, neuronal cell, or lymphocyte) by expression of a recombinant nucleic acid encoding an IAP polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes. By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The

term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) an IAP polypeptide.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell which includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic mammalian (e.g., rodents such as rats or mice) and the DNA (transgene) is inserted by artifice into the nuclear genome.

By "transformation" is meant any method for introducing foreign molecules into a cell. Lipofection, calcium phosphate precipitation, retroviral delivery, electroporation, and biolistic transformation are just a few of the teachings which may be used. For example, biolistic transformation is a method for introducing foreign molecules into a cell using velocity driven microprojectiles such as tungsten or gold particles. Such velocity-driven methods originate from

pressure bursts which include, but are not limited to, helium-driven, air-driven, and gunpowder-driven techniques. Biolistic transformation may be applied to the transformation or transfection of a wide variety of cell types and intact tissues including, without limitation, intracellular organelles (e.g., and mitochondria and chloroplasts), bacteria, yeast, fungi, algae, animal tissue, and cultured cells.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., an IAP polypeptide, a recombinant protein or a RNA molecule).

By "reporter gene" is meant a gene whose expression may be assayed; such genes include, without limitation, glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), and *lacZ*.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins are bound to the regulatory sequences).

By "conserved region" is meant any stretch of six or more contiguous amino acids exhibiting at least 30%, preferably 50%, and most preferably 70% amino acid sequence identity between two or more of the IAP family members, (e.g., between human HIAP-1, HIAP-2, and XIAP). Examples of preferred conserved regions are shown (as boxed or designated sequences) in Figures 5-7

and Tables 1 and 2, and include, without limitation, BIR domains and ring zinc finger domains.

By "detectably-labelled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule. Methods for detectably-labelling a molecule are well known in the art and include, without limitation, radioactive labelling (e.g., with an isotope such as ^{32}P or ^{35}S) and nonradioactive labelling (e.g., chemiluminescent labelling, e.g., fluorescein labelling).

By "antisense," as used herein in reference to nucleic acids, is meant a nucleic acid sequence, regardless of length, that is complementary to the coding strand of a gene.

By "purified antibody" is meant antibody which is at least 60%, by weight, free from proteins and naturally occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably 90%, and most preferably at least 99%, by weight, antibody, e.g., an IAP specific antibody. A purified antibody may be obtained, for example, by affinity chromatography using recombinantly-produced protein or conserved motif peptides and standard techniques.

By "specifically binds" is meant an antibody that recognizes and binds a protein but that does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, that naturally includes protein.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Fig. 1 is the human *xiap* cDNA sequence (SEQ ID NO: 3) and the XIAP polypeptide sequence (SEQ ID NO: 4).

Fig. 2 is the human *hiap-1* cDNA sequence (SEQ ID NO: 5) and the HIAP-1 polypeptide sequence (SEQ ID NO: 6).

Fig. 3 is the human *hiap-2* cDNA sequence (SEQ ID NO: 7) and the HIAP-2 polypeptide sequence (SEQ ID NO: 8). The sequence absent in the *hiap-2-Δ* variant is boxed.

Fig. 4 is the murine *xiap* cDNA sequence (SEQ ID NO: 9) and encoded murine XIAP polypeptide sequence (SEQ ID NO: 10).

Fig. 5 is the murine *hiap-1* cDNA sequence (SEQ ID NO: 39) and the encoded murine HIAP-1 polypeptide sequence (SEQ ID NO: 40).

Fig. 6 is the murine *hiap-2* cDNA sequence (SEQ ID NO: 41) and the encoded murine HIAP-2 polypeptide (SEQ ID NO: 42).

Fig. 7 is a representation of the alignment of the BIR domains of IAP proteins (SEQ ID NOs: 11 and 14-31).

Fig. 8 is a representation of the alignment of human IAP polypeptides with diap, cp-iap, and the IAP consensus sequence (SEQ ID NOs: 4, 6, 8, 10, 12, and 13).

Fig. 9 is a representation of the alignment of the ring zinc finger domains of IAP proteins (SEQ ID NOs: 32-38).

Fig. 10 is a photograph of a northern blot illustrating human *hiap-1* and *hiap-2* mRNA expression in human tissues.

Fig. 11 is a photograph of a northern blot illustrating human *hiap-2* mRNA expression in human tissues.

Fig. 12 is a photograph of a northern blot illustrating human *xiap* mRNA expression in human tissues.

Fig. 13A and 13B are photographs of agarose gels illustrating apoptotic DNA ladders and RT-PCR products using *hiap-1* and *hiap-2* specific probes in HIV-infected T cells.

Fig. 14A - 14D are graphs depicting suppression of apoptosis by XIAP, HIAP-1, HIAP-2, Bcl-2, smn, and 6-myc.

Fig. 15A and 15B are bar graphs depicting the percentage of viable CHO cells following transient transfection with the cDNA constructs shown and subsequent serum withdrawal.

Fig. 16A and 16B are bar graphs depicting the percentage of viable CHO cells following transient transfection with the cDNA constructs shown and subsequent exposure to menadione (Fig. 16A = 10 μM menadione; Fig. 16B = 20 μM menadione).

Fig. 17 is a photograph of an agarose gel containing cDNA fragments that were amplified, with *hiap-1*-specific primers, from RNA obtained from Raji, Ramos, EB-3, and Jiyoye cells, and from normal placenta.

Fig. 18 is a photograph of a western blot containing protein extracted from Jurkat and astrocytoma cells stained with an anti-XIAP antibody. The position and size of a series of marker proteins is indicated.

Fig. 19 is a photograph of a western blot containing protein extracted from Jurkat cells following treatment as described in Example XII. The blot was stained with a rabbit polyclonal anti-XIAP antibody. Lane 1, negative control; lane 2, anti-Fas antibody; lane 3, anti-Fas antibody and cycloheximide; lane 4, TNF-α; lane 5, TNF-α and cycloheximide.

Fig. 20 is a photograph of a western blot containing protein extracted from HeLa cells following exposure to anti-Fas antibodies. The blot was stained with a rabbit polyclonal anti-XIAP antibody. Lane 1, negative control; lane 2, cycloheximide; lane 3, anti-Fas antibody; lane 4, anti-Fas antibody and cycloheximide; lane 5, TNF-α; lane 6, TNF-α and cycloheximide.

Fig. 21A and 21B are photographs of western blots stained with rabbit polyclonal anti-XIAP antibody. Protein was extracted from HeLa cells (Fig. 21A) and Jurkat cells (Fig. 21B) immediately, 1, 2, 3, 5, 10, and 22 hours after exposure to anti-Fas antibody.

Fig. 22A and 22B are photographs of western blots stained with an anti-CPP32 antibody (Fig. 22A) or a rabbit polyclonal anti-XIAP antibody (Fig. 22B). Protein was extracted from Jurkat cells immediately, 3 hours, or 7 hours after exposure to an anti-Fas antibody. In addition to total protein, cytoplasmic and nuclear extracts are shown.

Fig. 23 is a photograph of a polyacrylamide gel following electrophoresis of the products of an *in vitro* XIAP cleavage assay.

Detailed Description

I. IAP Genes and Polypeptides

A new class of mammalian proteins that modulate apoptosis (IAPs) and the genes that encode these proteins have been discovered. The IAP proteins are characterized by the presence of a ring zinc finger domain (RZF; Fig. 9) and at least one BIR domain, as defined by the boxed consensus sequences shown in Figs. 7 and 8, and by the sequence domains listed in Tables 1 and 2. As examples of novel IAP genes and proteins, the cDNA sequences and amino acid sequences for human IAPs (HIAP-1, HIAP-2, and XIAP) and a new murine inhibitor of

apoptosis, XIAP, are provided. Additional members of the mammalian IAP family (including homologs from other species and mutant sequences) may be isolated using standard cloning techniques and the conserved amino acid sequences, primers, and probes provided herein and known in the art. Furthermore, IAPs include those proteins lacking the ring zinc finger, as further described below.

TABLE 1
NUCLEOTIDE POSITION OF CONSERVED DOMAINS*

	BIR-1	BIR-2	BIR-3	Ring Zinc Finger
h-xiap	109 - 312	520 - 723	826 - 1023	1348 - 1485
m-xiap	202 - 405	613 - 816	916 - 1113	1438 - 1575
h-hiap-1	273 - 476	693 - 893	951 - 1154	1824 - 1961
m-hiap-1	251 - 453	670 - 870	928 - 1131	1795 - 1932
h-hiap-2	373 - 576	787 - 987	1042 - 1245	1915 - 2052
m-hiap-2	215 - 418	608 - 808	863 - 1066	1763 - 1876

*Positions indicated correspond to those shown in Figs. 1-4.

TABLE 2
AMINO ACID POSITION OF CONSERVED DOMAINS*

	BIR-1	BIR-2	BIR-3	Ring Zinc Finger
h-XAIP	26 - 93	163 - 230	265 - 330	439 - 484
m-XIAP	26 - 93	163 - 230	264 - 329	438 - 483
h-HIAP1	29 - 96	169 - 235	255 - 322	546 - 591
m-HIAP1	29 - 96	169 - 235	255 - 322	544 - 589
h-HIAP2	46 - 113	184 - 250	269 - 336	560 - 605
m-HIAP2	25 - 92	156 - 222	241 - 308	541 - 578

*Positions indicated correspond to those shown in Figs. 1-4.

Recognition of the mammalian IAP family has provided an emergent pattern of protein structure. Recognition of this pattern allows proteins having a known, homologous sequence but unknown function to be classified as putative inhibitors of apoptosis. A *Drosophila* gene, now termed *diap*, was classified in this way (for sequence information see Genbank Accession Number M96581 and Fig. 6). The conservation of these proteins across species indicates that the apoptosis signalling pathway has been conserved throughout evolution.

The IAP proteins may be used to inhibit the apoptosis that occurs as part of numerous disease processes or disorders. For example, IAP polypeptides or nucleic acid encoding IAP polypeptides may be administered for the treatment or prevention of apoptosis that occurs as a part of AIDS, neurodegenerative diseases, ischemic injury, toxin-induced liver disease and myelodysplastic syndromes. Nucleic acid encoding the IAP polypeptide may also be provided to inhibit apoptosis.

II. Cloning of IAP Genes

A. Human *xiap*

The search for human genes involved in apoptosis resulted in the identification of an X-linked sequence tag site (STS) in the GenBank database, which demonstrated strong homology with the conserved RZF domain of CpIAP and OpIAP, the two baculovirus genes known to inhibit apoptosis (Clem et al., Mol. Cell Biol. 14:5212, 1994; Birnbaum et al., J. Virol. 68:2521, 1994). Screening a human fetal brain ZapII cDNA library (Stratagene, La Jolla, CA) with this STS resulted in the identification and cloning of *xiap* (for X-linked Inhibitor of Apoptosis Protein gene). The human gene has a 1.5 kb coding sequence that includes three BIR domains (Crook et al., J. Virol. 67:2168, 1993; Clem et al.,

Science 254:1388, 1991; Birnbaum et al., J. Virol. 68:2521, 1994) and a zinc finger. Northern blot analysis with *xiap* revealed message greater than 7 kb, which is expressed in various tissues, particularly liver and kidney (Fig. 12). The large size of the transcript reflects large 5' and 3' untranslated regions.

B. Human *hiap-1* and *hiap-2*

The *hiap-1* and *hiap-2* genes were cloned by screening a human liver library (Stratagene Inc., LaJolla, CA) with a probe including the entire *xiap* coding region at low stringency (the final wash was performed at 40°C with 2X SSC, 10% SDS; Figs. 2 and 3). The *hiap-1* and *hiap-2* genes were also detected independently using a probe derived from an expressed sequence tag (EST; GenBank Accession No. T96284), which includes a portion of a BIR domain. The EST sequence was originally isolated by the polymerase chain reaction; a cDNA library was used as a template and amplified with EST-specific primers. The DNA amplified probe was then used to screen the human liver cDNA library for full-length *hiap* coding sequences. A third DNA was subsequently detected that includes the *hiap-2* sequence but that appears to lack one exon, presumably due to alternative mRNA splicing (see boxed region in Fig. 3). The expression of *hiap-1* and *hiap-2* in human tissues as assayed by northern blot analysis is shown in Figs. 8 and 9.

C. *m-xiap*

Fourteen cDNA and two genomic clones were identified by screening a mouse embryo λgt11 cDNA library (Clontech, Palo Alto, CA) and a mouse FIX II genomic library with a *xiap* cDNA probe, respectively. A cDNA contig spanning 8.0 kb was constructed using 12 overlapping mouse clones. Sequence

analysis revealed a coding sequence of approximately 1.5 kb. The mouse gene, *m-xiap*, encodes a polypeptide with striking homology to human XIAP at and around the initiation methionine, the stop codon, the three BIR domains, and the RZF domain. As with the human gene, the mouse homologue contains large 5' and 3' UTRs, which could produce a transcript as large as 7-8 kb.

Analysis of the sequence and restriction map of *m-xiap* further delineate the structure and genomic organization of *m-xiap*. Southern blot analysis and inverse PCR techniques (Groden et al., Cell 66:589, 1991) can be employed to map exons and define exon-intron boundaries.

Antisera can be raised against a M-XIAP fusion protein that was obtained from, for example, *E. coli* using a bacterial expression system. The resulting antisera can be used along with northern blot analysis to analyze the spatial and temporal expression of *m-xiap* in the mouse.

D. *m-hiap-1* and *m-hiap-2*

The murine homologs of *hiap-1* and *hiap-2* were cloned and sequenced in the same general manner as m-xiap using the human *hiap-1* and *hiap-2* sequences as probes. Cloning of *m-hiap-1* and *m-hiap-2* further demonstrate that homologs from different species may be isolated using the techniques provided herein and those generally known to artisans skilled in molecular biology.

III. Identification of Additional IAP Genes

Standard techniques, such as the polymerase chain reaction (PCR) and DNA hybridization, may be used to clone additional human IAP genes and their homologues in other species. Southern blots of human genomic DNA

hybridized at low stringency with probes specific for *xiap*, *hiap-1* and *hiap-2* reveal bands that correspond to other known human IAP sequences as well as additional bands that do not correspond to known IAP sequences. Thus, additional IAP sequences may be readily identified using low stringency hybridization. Examples of murine and human *xiap*, *hiap-1*, and *hiap-2* specific primers, which may be used to clone additional genes by RT-PCR, are shown in Table 5.

IV. Characterization of IAP Activity and Intracellular Localization Studies

The ability of putative IAPs to modulate apoptosis can be defined in *in vitro* systems in which alterations of apoptosis can be detected. Mammalian expression constructs carrying IAP cDNAs, which are either full-length or truncated, can be introduced into cell lines such as CHO, NIH 3T3, HL60, Rat-1, or Jurkat cells. In addition, Sf2l insect cells may be used, in which case the IAP gene is preferentially expressed using an insect heat shock promotor. Following transfection, apoptosis can be induced by standard methods, which include serum withdrawal, or application of staurosporine, menadione (which induces apoptosis via free radial formation), or anti-Fas antibodies. As a control, cells are cultured under the same conditions as those induced to undergo apoptosis, but either not transfected, or transfected with a vector that lacks an IAP insert. The ability of each IAP construct to inhibit apoptosis upon expression can be quantified by calculating the survival index of the cells, i.e., the ratio of surviving transfected cells to surviving control cells. These experiments can confirm the presence of apoptosis inhibiting activity and, as discussed below, can also be used to determine the functional region(s) of an IAP. These assays may also be performed in combination with the application of additional compounds in order to identify compounds that modulate apoptosis via IAP expression.

A. Cell Survival Following Transfection with Full-length IAP Constructs and Induction of Apoptosis

Specific examples of the results obtained by performing various apoptosis suppression assays are shown in Figs. 14A to 14D. For example, CHO cell survival following transfection with one of six constructs and subsequent serum withdrawal is shown in Fig. 14A. The cells were transfected using Lipofectace™ with 2 μg of one of the following recombinant plasmids: pCDNA3-6myc-xiap (xiap), pCDNA3-6myc-hiap-1 (hiap-1), pCDNA3-6myc-hiap-2 (hiap-2), pCDNA3-bcl-2 (bcl-2), pCDNA3-HA-smn (smn), and pCDNA3-6myc (6-myc). Oligonucleotide primers were synthesized to allow PCR amplification and cloning of the *xiap*, *hiap-1*, and *hiap-2* ORFs in pCDNA3 (Invitrogen). Each construct was modified to incorporate a synthetic myc tag encoding six repeats of the peptide sequence MEQKLISEEDL (SEQ ID NO: 43), thus allowing detection of myc-IAP fusion proteins via monoclonal anti-myc antiserum (Egan et al., Nature 363:45, 1993). Triplicate samples of cell lines in 24-well dishes were washed 5 times with serum free media and maintained in serum free conditions during the course of the experiment. Cells that excluded trypan blue, and that were therefore viable, were counted with a hemocytometer immediately, 24 hours, 48 hours, and 72 hours after serum withdrawal. Survival was calculated as a percentage of the initial number of viable cells. In this experiment, as well as those presented in Figs. 14B and 14D, the percentage of viable cells shown represents the average of three separate experiments performed in triplicate, ± standard deviation.

The survival of CHO cells following transfection (with each one of the six constructs described above) and exposure to menadione is shown in Fig. 14B. The cells were plated in 24-well dishes, allowed to grow overnight, and then

exposed to 20 μM menadione (Sigma Chemical Co., St. Louis, MO) for 1.5 hours. Triplicate samples were harvested at the time of exposure to menadione and 24 hours afterward, and survival was assessed by trypan blue exclusion.

The survival of Rat-1 cells following transfection (with each one of the six constructs described above) and exposure to staurosporine is shown in Fig. 14C. Rat-1 cells were transfected and then selected in medium containing 800 μg/ml G418 for two weeks. The cell line was assessed for resistance to staurosporine-induced apoptosis (1 μM) for 5 hours. Viable cells were counted 24 hours after exposure to staurosporine by trypan blue exclusion. The percentage of viable cells shown represents the average of two experiments, ± standard deviation.

The Rat-1 cell line was also used to test the resistance of these cells to menadione (Fig. 14D) following transfection with each of the six constructs described above. The cells were exposed to 10 μM menadione for 1.5 hours, and the number of viable cells was counted 18 hours later.

B. Comparison of Cell Survival FollowingTransfection with Full-length vs. Partial IAP Constructs

In order to investigate the mechanism whereby human IAPs, including XIAP, HIAP-1, and HIAP-2, afford protection against cell death, expression vectors were constructed that contained either: (1) full-length IAP cDNA (as described above), (2) a portion of an IAP gene that encodes the BIR domains, but not the RZF, or (3) a portion of an IAP gene that encodes the RZF, but not the BIR domains. Human and murine *xiap* or *m-xiap* cDNAs were tested by transient or stable expression in HeLa, Jurkat, and CHO cell lines. Following transfection, apoptosis was induced by serum withdrawal, application of

menadione, or application of an anti-Fas antibody. Cell death was then assessed, as described above, by trypan blue exclusion. As a control for transfection efficiency, the cells were co-transfected with a β-gal expression construct. Typically, approximately 20% of the cells were successfully transfected.

When CHO cells were transiently transfected, constructs containing full-length *xiap* or *m-xiap* cDNAs conferred modest protection against cell death (Fig. 15A). In contrast, the survival of CHO cells transfected with constructs encoding only the BIR domains (i.e., lacking the RZF domain; see Fig. 15A) was markedly enhanced 72 hours after serum deprivation. Furthermore, a large percentage of cells expressing the BIR domains were still viable after 96 hours, at which time no viable cells remained in the control, i.e. non-transfected, cell cultures (see "CHO" in Fig. 15A), and less than 5% of the cells transfected with the vector only, i.e., lacking a cDNA insert, remained viable (see "pcDNA3" in Fig. 15A). Deletion of any of the BIR domains results in the complete loss of apoptotic suppression, which is reflected by a decrease in the percentage of surviving CHO cells to control levels within 72 hours of serum withdrawal (Fig. 15B; see "xiapΔ1" (which encodes amino acids 89-497 of XIAP (SEQ ID NO.:4)), "xiapΔ2" (which encodes amino acids 246-497 of XIAP (SEQ ID NO.:4)), and "xiapΔ3" (which encodes amino acids 342-497 of XIAP (SEQ ID NO.:4)) at 72 hours).

Stable pools of transfected CHO cells, which were maintained for several months under G418 selection, were induced to undergo apoptosis by exposure to 10 μM menadione for 2 hours. Among the CHO cells tested were those that were stably transfected with: (1) full-length *m-xiap* cDNA (miap), (2) full-length *xiap* cDNA (xiap), (3) full-length *bcl-2* cDNA (Bcl-2), (4) cDNA encoding the three BIR domains (but not the RZF) of M-XIAP (BIR), and (5)

cDNA encoding the RZF (but not BIR domains) of M-XIAP (RZF). Cells that were non-transfected (CHO) or transfected with the vector only (pcDNA3), served as controls for this experiment. Following exposure to 10 μM menadione, the transfected cells were washed with phosphate buffered saline (PBS) and cultured for an additional 24 hours in menadione-free medium. Cell death was assessed, as described above, by trypan blue exclusion. Less than 10% of the non-transfected or vector-only transfected cells remained viable at the end of the 24 hour survival period. Cells expressing the RZF did not fare significantly better. However, expression of full-length *m-xiap*, *xiap*, or *bcl-2*, and expression of the BIR domains, enhanced cell survival (Fig. 16A). When the concentration of menadione was increased from 10 μM to 20 μM (with all other conditions of the experiment being the same as when 10 μM menadione was applied), the percentage of viable CHO cells that expressed the BIR domain cDNA construct was higher than the percentage of viable cells that expressed either full-length *m-xiap* or *bcl-2* (Fig. 16B).

C. Analysis of the Subcellular Location of Expressed RZF and BIR Domains

The assays of cell death described above indicate that the RZF may act as a negative regulator of the anti-apoptotic function of IAPs. One way in which the RZF, and possibly other IAP domains, may exert their regulatory influence is by altering the expression of genes, whose products function in the apoptotic pathway.

In order to determine whether the subcellular locations of expressed RZF and BIR domains are consistent with roles as nuclear regulatory factors, COS cells were transiently transfected with the following four constructs, and the expressed polypeptide was localized by immunofluorescence microscopy: (1)

pcDNA3-6myc-xiap, which encodes all 497 amino acids of SEQ ID NO: 4, (2) pcDNA3-6myc-m-xiap, which encodes all 497 amino acids of mouse *xiap* (SEQ ID NO: 10), (3) pcDNA3-6myc-mxiap-BIR, which encodes amino acids 1 to 341 of *m-xiap* (SEQ ID NO: 10), and (4) pcDNA3-6myc-mxiap-RZF, which encodes amino acids 342-497 of *m-xiap* (SEQ ID NO: 10). The cells were grown on multi-well tissue culture slides for 12 hours, and then fixed and permeabilized with methanol. The constructs used (here and in the cell death assays) were tagged with a human Myc epitope tag at the N-terminus. Therefore, a monoclonal anti-Myc antibody and a secondary goat anti-mouse antibody, which was conjugated to FITC, could be used to localize the expressed products in transiently transfected COS cells. Full-length XIAP and MIAP were located in the cytoplasm, with accentuated expression in the peri-nuclear zone. The same pattern of localization was observed when the cells expressed a construct encoding the RZF domain (but not the BIR domains). However, cells expressing the BIR domains (without the RZF) exhibited, primarily, nuclear staining. The protein expressed by the BIR domain construct appeared to be in various stages of transfer to the nucleus.

These observations are consistent with the fact that, as described below, XIAP is cleaved within T cells that are treated with anti-Fas antibodies (which are potent inducers of apoptosis), and its N-terminal domain is translocated to the nucleus.

D. Examples of Additional Apoptosis Assays

Specific examples of apoptosis assays are also provided in the following references. Assays for apoptosis in lymphocytes are disclosed by: Li et al., Science 268:429, 1995; Gibellini et al., Br. J. Haematol. 89:24, 1995; Martin et

al., J. Immunol. 152:330, 1994; Terai et al., J. Clin. Invest. 87:1710, 1991; Dhein et al., Nature 373:438, 1995; Katsikis et al., J. Exp. Med. 1815:2029, 1995; Westendorp et al., Nature 375:497, 1995; DeRossi et al., Virology 198:234, 1994.

Assays for apoptosis in fibroblasts are disclosed by: Vossbeck et al., Int. J. Cancer 61:92, 1995; Goruppi et al., Oncogene 9:1537, 1994; Fernandez et al., Oncogene 9:2009, 1994; Harrington et al., EMBO J., 13:3286, 1994; Itoh et al., J. Biol. Chem. 268:10932, 1993.

Assays for apoptosis in neuronal cells are disclosed by: Melino et al., Ann. Neurol. 36:864, 1994; Sato et al., J. Neurobiol. 25:1227, 1994; Ferrari et al., J. Neurosci. 1516:2857, 1995; Talley et al., Mol. Cell Biol. 1585:2359, 1995; Talley et al., Mol. Cell. Biol. 15:2359, 1995; Walkinshaw et al., J. Clin. Invest. 95:2458, 1995.

Assays for apoptosis in insect cells are disclosed by: Clem et al., Science 254:1388, 1991; Crook et al., J. Virol. 67:2168, 1993; Rabizadeh et al., J. Neurochem. 61:2318, 1993; Birnbaum et al., J. Virol. 68:2521, 1994; Clem et al., Mol. Cell. Biol. 14:5212, 1994.

V. Construction of a Transgenic Animal

Characterization of IAP genes provides information that is necessary for an IAP knockout animal model to be developed by homologous recombination. Preferably, the model is a mammalian animal, most preferably a mouse. Similarly, an animal model of IAP overproduction may be generated by integrating one or more IAP sequences into the genome, according to standard transgenic techniques.

A replacement-type targeting vector, which would be used to create a knockout model, can be constructed using an isogenic genomic clone, for

example, from a mouse strain such as 129/Sv (Stratagene Inc., LaJolla, CA). The targeting vector is introduced into a suitably-derived line of embryonic stem (ES) cells by electroporation to generate ES cell lines that carry a profoundly truncated form of an IAP. To generate chimeric founder mice, the targeted cell lines are injected into a mouse blastula stage embryo. Heterozygous offspring are interbred to homozygosity. Knockout mice would provide the means, *in vivo*, to screen for therapeutic compounds that modulate apoptosis via an IAP-dependent pathway.

VI. IAP Protein Expression

IAP genes may be expressed in both prokaryotic and eukaryotic cell types. If an IAP modulates apoptosis by exacerbating it, it may be desirable to express that protein under control of an inducible promotor.

In general, IAPs according to the invention may be produced by transforming a suitable host cell with all or part of an IAP-encoding cDNA fragment that has been placed into a suitable expression vector.

Those skilled in the art of molecular biology will understand that a wide variety of expression systems may be used to produce the recombinant protein. The precise host cell used is not critical to the invention. The IAP protein may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *S. cerevisiae*, insect cells such as Sf2l cells, or mammalian cells such as COS-1, NIH 3T3, or HeLa cells). These cells are publicly available, for example, from the American Type Culture Collection (ATCC), Rockville, MD; see also Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1994. The method of transduction and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*supra*), and expression vehicles may be chosen

from those provided, e.g. in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels et al., 1985, Supp. 1987).

A preferred expression system is the baculovirus system using, for example, the vector pBacPAK9, which is available from Clontech (Palo Alto, CA). If desired, this system may be used in conjunction with other protein expression techniques, for example, the myc tag approach described by Evan et al. (Mol. Cell Biol. 5:3610, 1985).

Alternatively, an IAP may be produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, (e.g., see Pouwels et al., *supra*), as are methods for constructing such cell lines (e.g., see Ausubel et al., *supra*). In one example, cDNA encoding an IAP is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, integration of the IAP-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (as described in Ausubel et al., *supra*). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene.

Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (*supra*). These methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. The most commonly used DHFR-containing expression vectors are pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., *supra*). The host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR$^-$ cells, ATCC Accession No. CRL 9096) are among those most preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

Once the recombinant protein is expressed, it is isolated by, for example, affinity chromatography. In one example, an anti-IAP antibody, which may be produced by the methods described herein, can be attached to a column and used to isolate the IAP protein. Lysis and fractionation of IAP-harboring cells prior to affinity chromatography may be performed by standard methods (see e.g., Ausubel et al., *supra*). Once isolated, the recombinant protein can, if desired, be purified further by e.g., by high performance liquid chromatography (HPLC; e.g., see Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, Work and Burdon, Eds., Elsevier, 1980).

Polypeptides of the invention, particularly short IAP fragments, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL). These general techniques of polypeptide expression and purification can also be used to produce and isolate useful IAP fragments or analogs, as described herein.

VII. Anti-IAP Antibodies

In order to generate IAP-specific antibodies, an IAP coding sequence (i.e., amino acids 180-276) can be expressed as a C-terminal fusion with glutathione S-transferase (GST; Smith et al., Gene 67:31, 1988). The fusion protein can be purified on glutathione-Sepharose beads, eluted with glutathione, and cleaved with thrombin (at the engineered cleavage site), and purified to the degree required to successfully immunize rabbits. Primary immunizations can be carried out with Freund's complete adjuvant and subsequent immunizations performed with Freund's incomplete adjuvant. Antibody titres are monitored by western blot and immunoprecipitation analyses using the thrombin-cleaved IAP

fragment of the GST-IAP fusion protein. Immune sera are affinity purified using CNBr-Sepharose-coupled IAP protein. Antiserum specificity is determined using a panel of unrelated GST proteins (including GSTp53, Rb, HPV-16 E6, and E6-AP) and GST-trypsin (which was generated by PCR using known sequences).

As an alternate or adjunct immunogen to GST fusion proteins, peptides corresponding to relatively unique hydrophilic regions of IAP may be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides is similarly affinity purified on peptides conjugated to BSA, and specificity is tested by ELISA and western blotting using peptide conjugates, and by western blotting and immunoprecipitation using IAP expressed as a GST fusion protein.

Alternatively, monoclonal antibodies may be prepared using the IAP proteins described above and standard hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In *Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, New York, NY, 1981; Ausubel et al., *supra*). Once produced, monoclonal antibodies are also tested for specific IAP recognition by western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., *supra*).

Antibodies that specifically recognize IAPs or fragments of IAPs, such as those described herein containing one or more BIR domains (but not a ring zinc finger domain), or that contain a ring zinc finger domain (but not a BIR domain) are considered useful in the invention. They may, for example, be used in an immunoassay to monitor IAP expression levels or to determine the subcellular location of an IAP or IAP fragment produced by a mammal. Antibodies that inhibit the 26 kDa IAP cleavage product described herein (which

contains at least one BIR domain) may be especially useful in inducing apoptosis in cells undergoing undesirable proliferation.

Preferably, antibodies of the invention are produced using IAP sequence that does not reside within highly conserved regions, and that appears likely to be antigenic, as analyzed by criteria such as those provided by the Peptide structure program (Genetics Computer Group Sequence Analysis Package, Program Manual for the GCG Package, Version 7, 1991) using the algorithm of Jameson and Wolf (CABIOS 4:181, 1988). Specifically, these regions, which are found between BIR1 and BIR2 of all IAPs, are: amino acid 99 to amino acid 170 of HIAP-1, amino acid 123 to amino acid 184 of HIAP-2, and amino acid 116 to amino acid 133 of either XIAP or M-XIAP. These fragments can be generated by standard techniques, e.g., by PCR, and cloned into the pGEX expression vector (Ausubel et al., *supra*). Fusion proteins are expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel et al. (*supra*). In order to minimize the potential for obtaining antisera that is non-specific, or exhibits low-affinity binding to IAP, two or three fusions are generated for each protein, and each fusion is injected into at least two rabbits. Antisera are raised by injections in series, preferably including at least three booster injections.

VIII. Identification of Molecules that Modulate IAP Protein Expression

Isolation of IAP cDNAs also facilitates the identification of molecules that increase or decrease IAP expression. In one approach, candidate molecules are added, in varying concentration, to the culture medium of cells expressing IAP mRNA. IAP expression is then measured, for example, by northern blot analysis (Ausubel et al., *supra*) using an IAP cDNA, or cDNA fragment, as a hybridization probe (see also Table 5). The level of IAP expression

in the presence of the candidate molecule is compared to the level of IAP expression in the absence of the candidate molecule, all other factors (e.g. cell type and culture conditions) being equal.

The effect of candidate molecules on IAP-mediated apoptosis may, instead, be measured at the level of translation by using the general approach described above with standard protein detection techniques, such as western blotting or immunoprecipitation with an IAP-specific antibody (for example, the IAP antibody described herein).

Compounds that modulate the level of IAP may be purified, or substantially purified, or may be one component of a mixture of compounds such as an extract or supernatant obtained from cells (Ausubel et al., *supra*). In an assay of a mixture of compounds, IAP expression is tested against progressively smaller subsets of the compound pool (e.g., produced by standard purification techniques such as HPLC or FPLC) until a single compound or minimal number of effective compounds is demonstrated to modulate IAP expression.

Compounds may also be screened for their ability to modulate IAP apoptosis inhibiting activity. In this approach, the degree of apoptosis in the presence of a candidate compound is compared to the degree of apoptosis in its absence, under equivalent conditions. Again, the screen may begin with a pool of candidate compounds, from which one or more useful modulator compounds are isolated in a step-wise fashion. Apoptosis activity may be measured by any standard assay, for example, those described herein.

Another method for detecting compounds that modulate the activity of IAPs is to screen for compounds that interact physically with a given IAP polypeptide. These compounds may be detected by adapting interaction trap expression systems known in the art. These systems detect protein interactions

using a transcriptional activation assay and are generally described by Gyuris et al. (Cell 75:791, 1993) and Field et al. (Nature 340:245, 1989), and are commercially available from Clontech (Palo Alto, CA). In addition, PCT Publication WO 95/28497 describes an interaction trap assay in which proteins involved in apoptosis, by virtue of their interaction with Bcl-2, are detected. A similar method may be used to identify proteins and other compounds that interact with IAPs.

Compounds or molecules that function as modulators of IAP-mediated cell death may include peptide and non-peptide molecules such as those present in cell extracts, mammalian serum, or growth medium in which mammalian cells have been cultured.

A molecule that promotes an increase in IAP expression or IAP activity is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to increase cellular levels of IAP and thereby exploit the ability of IAP polypeptides to inhibit apoptosis.

A molecule that decreases IAP activity (e.g., by decreasing IAP gene expression or polypeptide activity) may be used to decrease cellular proliferation. This would be advantageous in the treatment of neoplasms (see Table 3, below), or other cell proliferative diseases.

TABLE 3
NORTHERN BLOT IAP RNA LEVELS IN CANCER CELLS*

	xiap	*hiap1*	*hiap2*
Promyelocytic Leukemia HL-60	+	+	+
Hela S-3	+	+	+
Chronic Myelogenous Leukemia K-562	+++	+	+++
Lymphoblastic Leukemia MOLT-4	+++	+	+
Burkitt's Lymphoma Raji	+	+(x10)	+
Colorectal Adenocarcinoma SW-480	+++	+++	+++
Lung Carcinoma A-549	+	+	+
Melanoma G-361	+++	+	+

*Levels are indicated by a (+) and are the approximate increase in RNA levels relative to northern blots of RNA from non-cancerous control cell lines. A single plus indicates an estimated increase of at least 1-fold

Molecules that are found, by the methods described above, to effectively modulate IAP gene expression or polypeptide activity may be tested further in animal models. If they continue to function successfully in an *in vivo* setting, they may be used as therapeutics to either inhibit or enhance apoptosis, as appropriate.

IX. IAP Therapy

The level of IAP gene expression correlates with the level of apoptosis. Thus, IAP genes also find use in anti-apoptosis gene therapy. In particular, a functional IAP gene may be used to sustain neuronal cells that undergo apoptosis in the course of a neurodegenerative disease, lymphocytes (i.e., T cells and B cells), or cells that have been injured by ischemia.

Retroviral vectors, adenoviral vectors, adeno-associated viral vectors, or other viral vectors with the appropriate tropism for cells likely to be involved in

apoptosis (for example, epithelial cells) may be used as a gene transfer delivery system for a therapeutic IAP gene construct. Numerous vectors useful for this purpose are generally known (Miller, Human Gene Therapy 15, 1990; Friedman, Science 244:1275, 1989; Eglitis and Anderson, Biotechniques 6:608, 1988; Tolstoshev and Anderson, Curr. Opin. Biotechnol. 1:55, 1990; Sharp, Lancet 337:1277, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311, 1987; Anderson, Science 226:401, 1984; Moen, Blood Cells 17:407, 1991; Miller et al., Biotechniques 7:980, 1989; La Salle et al., Science 259:988, 1993; Johnson, Chest 107:77S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med. 323:370, 1990; Anderson et al., U.S. Patent No. 5,399,346). Non-viral approaches may also be employed for the introduction of therapeutic DNA into cells otherwise predicted to undergo apoptosis. For example, IAP may be introduced into a neuron or a T cell by lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413, 1987; Ono et al., Neurosci. Lett. 117:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Meth. Enzymol. 101:512, 1983), asialorosonucoid-polylysine conjugation (Wu et al., J. Biol. Chem. 263:14621, 1988; Wu et al., J. Biol. Chem. 264:16985, 1989); or, less preferably, microinjection under surgical conditions (Wolff et al., Science 247:1465, 1990).

For any of the methods of application described above, the therapeutic IAP DNA construct is preferably applied to the site of the predicted apoptosis event (for example, by injection). However, it may also be applied to tissue in the vicinity of the predicted apoptosis event or to a blood vessel supplying the cells predicted to undergo apoptosis.

In the constructs described, IAP cDNA expression can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus

40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in neural cells, T cells, or B cells may be used to direct IAP expression. The enhancers used could include, without limitation, those that are characterized as tissue- or cell-specific in their expression. Alternatively, if an IAP genomic clone is used as a therapeutic construct (for example, following its isolation by hybridization with the IAP cDNA described above), regulation may be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

Less preferably, IAP gene therapy is accomplished by direct administration of the IAP mRNA or antisense IAP mRNA to a cell that is expected to undergo apoptosis. The mRNA may be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using an IAP cDNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of IAP mRNA to malignant cells can be carried out by any of the methods for direct nucleic acid administration described above.

Ideally, the production of IAP protein by any gene therapy approach will result in cellular levels of IAP that are at least equivalent to the normal, cellular level of IAP in an unaffected cell. Treatment by any IAP-mediated gene therapy approach may be combined with more traditional therapies.

Another therapeutic approach within the invention involves administration of recombinant IAP protein, either directly to the site of a predicted apoptosis event (for example, by injection) or systemically (for example, by any conventional recombinant protein administration technique). The dosage of IAP depends on a number of factors, including the size and health of the individual

patient, but, generally, between 0.1 mg and 100 mg inclusive are administered per day to an adult in any pharmaceutically acceptable formulation.

X. Administration of IAP Polypeptides, IAP Genes, or Modulators of IAP Synthesis or Function

An IAP protein, gene, or modulator may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer IAP to patients suffering from a disease that is caused by excessive apoptosis. Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intraarterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for IAP modulatory compounds include ethylene-vinyl

acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

If desired, treatment with an IAP protein, gene, or modulatory compound may be combined with more traditional therapies for the disease such as surgery, steroid therapy, or chemotherapy for autoimmune disease; antiviral therapy for AIDS; and tissue plasminogen activator (TPA) for ischemic injury.

XI. Detection of Conditions Involving Altered Apoptosis

IAP polypeptides and nucleic acid sequences find diagnostic use in the detection or monitoring of conditions involving aberrant levels of apoptosis. For example, decrease expression of IAP may be correlated with enhanced apoptosis in humans (see section XII, below). Accordingly, a decrease or increase in the level of IAP production may provide an indication of a deleterious condition. Levels of IAP expression may be assayed by any standard technique. For example, IAP expression in a biological sample (e.g., a biopsy) may be monitored by standard northern blot analysis or may be aided by PCR (see, e.g., Ausubel et al., *supra*; *PCR Technology: Principles and Applications for DNA Amplification*, H.A. Ehrlich, Ed. Stockton Press, NY; Yap et al. Nucl. Acids. Res. 19:4294, 1991).

Alternatively, a biological sample obtained from a patient may be analyzed for one or more mutations in the IAP sequences using a mismatch detection approach. Generally, these techniques involve PCR amplification of nucleic acid from the patient sample, followed by identification of the mutation

(i.e., mismatch) by either altered hybridization, aberrant electrophoretic gel migration, binding or cleavage mediated by mismatch binding proteins, or direct nucleic acid sequencing. Any of these techniques may be used to facilitate mutant IAP detection, and each is well known in the art; examples of particular techniques are described, without limitation, in Orita et al., Proc. Natl. Acad. Sci. USA 86:2766, 1989; Sheffield et al., Proc. Natl. Acad. Sci. USA 86:232, 1989).

In yet another approach, immunoassays are used to detect or monitor IAP protein in a biological sample. IAP-specific polyclonal or monoclonal antibodies (produced as described above) may be used in any standard immunoassay format (e.g., ELISA, western blot, or RIA) to measure IAP polypeptide levels. These levels would be compared to wild-type IAP levels, with a decrease in IAP production indicating a condition involving increased apoptosis. Examples of immunoassays are described, e.g., in Ausubel et al., *supra*. Immunohistochemical techniques may also be utilized for IAP detection. For example, a tissue sample may be obtained from a patient, sectioned, and stained for the presence of IAP using an anti-IAP antibody and any standard detection system (e.g., one which includes a secondary antibody conjugated to horseradish peroxidase). General guidance regarding such techniques can be found in, e.g., Bancroft and Stevens (*Theory and Practice of Histological Techniques*, Churchill Livingstone, 1982) and Ausubel et al. (*supra*).

In one preferred example, a combined diagnostic method may be employed that begins with an evaluation of IAP protein production (for example, by immunological techniques or the protein truncation test (Hogerrorst et al., Nat. Gen. 10:208, 1995)) and also includes a nucleic acid-based detection technique designed to identify more subtle IAP mutations (for example, point mutations). As described above, a number of mismatch detection assays are available to those

skilled in the art, and any preferred technique may be used. Mutations in IAP may be detected that either result in loss of IAP expression or loss of IAP biological activity. In a variation of this combined diagnostic method, IAP biological activity is measured as protease activity using any appropriate protease assay system (for example, those described above).

Mismatch detection assays also provide an opportunity to diagnose an IAP-mediated predisposition to diseases caused by inappropriate apoptosis. For example, a patient heterozygous for an IAP mutation may show no clinical symptoms and yet possess a higher than normal probability of developing one or more types of neurodegenerative, myelodysplastic or ischemic diseases. Given this diagnosis, a patient may take precautions to minimize their exposure to adverse environmental factors (for example, UV exposure or chemical mutagens) and to carefully monitor their medical condition (for example, through frequent physical examinations). This type of IAP diagnostic approach may also be used to detect IAP mutations in prenatal screens. The IAP diagnostic assays described above may be carried out using any biological sample (for example, any biopsy sample or bodily fluid or tissue) in which IAP is normally expressed. Identification of a mutant IAP gene may also be assayed using these sources for test samples.

Alternatively, an IAP mutation, particularly as part of a diagnosis for predisposition to IAP-associated degenerative disease, may be tested using a DNA sample from any cell, for example, by mismatch detection techniques. Preferably, the DNA sample is subjected to PCR amplification prior to analysis.

In order to demonstrate the utility of IAP gene sequences as diagnostics and prognostics for cancer, a Human Cancer Cell Line Multiple Tissue Northern Blot (Clontech, Palo Alto, CA; #7757-1) was probed. This northern blot

contained approximately 2 μg of poly A^+ RNA per lane from eight different human cell lines: (1) promyelocytic leukemia HL-60, (2) HeLa cell S3, (3) chronic myelogenous leukemia K-562, (4) lymphoblastic leukemia MOLT-4, (5) Burkitt's lymphoma Raji, (6) colorectal adenocarcinoma SW480, (7) lung carcinoma A549, and (8) melanoma G361. As a control, a Human Multiple Tissue Northern Blot (Clontech, Palo Alto, CA; #7759-1) was probed. This northern blot contained approximately 2 μg of poly A^+ RNA from eight different human tissues: (1) spleen, (2) thymus, (3) prostate, (4) testis, (5) ovary, (6) small intestine, (7) colon, and (8) peripheral blood leukocytes.

The northern blots were hybridized sequentially with: (1) a 1.6 kb probe to the *xiap* coding region, (2) a 375 bp *hiap-2* specific probe corresponding to the 3' untranslated region, (3) a 1.3 kb probe to the coding region of *hiap-1*, which cross-reacts with *hiap-2*, (4) a 1.0 kb probe derived from the coding region of *bcl-2*, and (5) a probe to β-actin, which was provided by the manufacturer. Hybridization was carried out at 50°C overnight, according to the manufacturer's suggestion. The blot was washed twice with 2X SSC, 0.1% SDS at room temperature for 15 minutes and then with 2X SSC, 0.1% SDS at 50°C.

All cancer lines tested showed increased IAP expression relative to samples from non-cancerous control tissues (Table 3). Expression of *xiap* was particularly high in HeLa (S-3), chronic myelogenous leukemia (K-562), colorectal adenocarcinoma (SW-480), and melanoma (G-361) lines. Expression of *hiap-1* was extremely high in Burkitt's lymphoma, and was also elevated in colorectal adenocarcinoma. Expression of *hiap-2* was particularly high in chronic myelogenous leukemia (K-562) and colorectal adenocarcinoma (SW-480). Expression of *bcl-2* was upregulated only in HL-60 leukemia cells.

These observations suggest that upregulation of the anti-apoptotic IAP genes may be a widespread phenomenon, perhaps occurring much more frequently than upregulation of *bcl-2*. Furthermore, upregulation may be necessary for the establishment or maintenance of the transformed state of cancerous cells.

In order to pursue the observation described above, i.e., that *hiap-1* is overexpressed in the Raji Burkitt's lymphoma cell line, RT-PCR analysis was performed in multiple Burkitt's lymphoma cell lines. Total RNA was extracted from cells of the Raji, Ramos, EB-3, and Jiyoye cell lines, and as a positive control, from normal placental tissue. The RNA was reverse transcribed, and amplified by PCR with the following set of oligonucleotide primers: 5'-AGTGCGGGTTTTTATTATGTG-3' (SEQ ID NO: 44) and 5'-AGATGACCACAAGGAATAAACACTA-3' (SEQ ID NO: 45), which selectively amplify a *hiap-1* cDNA fragment. RT-PCR was conducted using a Perkin Elmer 480 Thermocycler to carry out 35 cycles of the following program: 94°C for 1 minute, 50°C for 1.5 minutes, and 72°C for a minute. The PCR reaction product was electrophoresed on an agarose gel and stained with ethidium bromide. Amplified cDNA fragments of the appropriate size were clearly visible in all lanes containing Burkitt's lymphoma samples, but absent in the lanes containing the normal placental tissue sample, and absent in lanes containing negative control samples, where template DNA was omitted from the reaction (Fig. 17).

XII. Accumulation of a 26 kDa Cleavage Protein in Astrocytoma Cells

A. Identification of a 26 kDa Cleavage Protein

A total protein extract was prepared from Jurkat and astrocytoma cells by sonicating them (X3 for 15 seconds at 4°C) in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin, and 5 mM benzamidine. Following sonication, the samples were centrifuged (14,000 RPM in a microfuge) for five minutes. Twenty μg of protein was loaded per well on a 10% SDS-polyacrylamide gel, electrophoresed, and electroblotted by standard methods to PVDF membranes. Western blot analysis, performed as described previously, revealed that the astrocytoma cell line (CCF-STTG1) abundantly expressed an anti-xiap reactive band of approximately 26 kDa, despite the lack of an apoptotic trigger event (Fig. 18). In fact, this cell line has been previously characterized as being particularly resistant to standard apoptotic triggers.

A 26 kDa XIAP-reactive band was also observed under the following experimental conditions. Jurkat cells (a transformed human T cell line) were induced to undergo apoptosis by exposure to an anti-Fas antibody (1 μg/ml). Identical cultures of Jurkat cells were exposed either to: (1) anti-Fas antibody and cycloheximide (20 μg/ml), (2) tumor necrosis factor alpha (TNF-α, at 1,000 U/ml), or (3) TNF-α and cycloheximide (20 μg/ml). All cells were harvested 6 hours after treatment began. In addition, as a negative control, anti-Fas antibody was added to an extract after the cells were harvested. The cells were harvested in SDS sample buffer, electrophoresed on a 12.5% SDS polyacrylamide gel, and electroblotted onto PVDF membranes using standard methods. The membranes were immunostained with a rabbit polyclonal anti-XIAP antibody at 1:1000 for 1 hour at room temperature. Following four 15 minute washes, a goat anti-rabbit antibody conjugated to horse-radish peroxidase was applied at room temperature

for 1 hour. Unbound secondary antibody was washed away, and chemiluminescent detection of XIAP protein was performed. The western blot revealed the presence of the full-length, 55 kDa XIAP protein, both in untreated and treated cells. In addition, a novel, approximately 26 kDa XIAP-reactive band was also observed in apoptotic cell extracts, but not in the control, untreated cell extracts (Fig. 19).

Cleavage of XIAP occurs in a variety of cell types, including other cancer cell lines such as HeLa. The expression of the 26 kDa XIAP cleavage product was demonstrated in HeLa cells as follows. HeLa cells were treated with either: (1) cyclohexamide (20 μg/ml), (2) anti-Fas antibody (1 μg/ml), (3) anti-Fas antibody (1 μg/ml) and cyclohexamide (20 μg/ml), (4) TNFα (1,000 U/ml), or (5) TNFα (1,000 U/ml) and cyclohexamide (20 μg/ml). All cells were harvested 18 hours after treatment began. As above, anti-Fas antibody was added to an extract after the cells were harvested. HeLa cells were harvested, and the western blot was probed under the same conditions as used to visualize xiap-reactive bands from Jurkat cell samples. A 26 kDa XIAP band was again seen in the apoptotic cell preparations (Fig. 20). Furthermore, the degree of XIAP cleavage correlated positively with the extent of apoptosis. Treatment of HeLa cells with cycloheximide or TNFα alone caused only minor apoptosis, and little cleavage product was observed. If the cells were treated with the anti-Fas antibody, a greater amount of cleavage product was apparent. These data indicate that XIAP is cleaved in more than one cell type and in response to more than one type of apoptotic trigger.

B. Time Course of Expression

The time course over which the 26 kDa cleavage product accumulates was examined by treating HeLa and Jurkat cells with anti-Fas antibody (1 μg/ml) and harvesting them either immediately, or 1, 2, 3, 5, 10, or 22 hours after treatment. Protein extracts were prepared and western blot analysis was performed as described above. Both types of cells accumulated increasing quantities of the 26 kDa cleavage product over the time course examined (Figs. 21A and 21B).

C. Subcellular Localization of the 26 kDa XIAP Cleavage Product

In order to determine the subcellular location of the 26 kDa cleavage product, Jurkat cells were induced to undergo apoptosis by exposure to anti-Fas antibody (1 μg/ml) and were then harvested either immediately, 3 hours, or 7 hours later. Total protein extracts were prepared, as described above, from cells harvested at each time point. In order to prepare nuclear and cytoplasmic cell extracts, apoptotic Jurkat cells were washed with isotonic Tris buffered saline (pH 7.0) and lysed by freezing and thawing five times in cell extraction buffer (50 mM PIPES, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, and 20 μM cytochalasin B). Nuclei were pelleted by centrifugation and resuspended in isotonic Tris (pH 7.0) and frozen at -80°C. The cytoplasmic fraction of the extract was processed further by centrifugation at 60,000 RPM in a TA 100.3 rotor for 30 minutes. Supernatants were removed and frozen at -80°C. Samples of both nuclear and cytoplasmic fractions were loaded on a 12.5% SDS-polyacrylamide gel, and electroblotted onto PVDF membranes. Western blot analysis was then performed using either an anti-CPP32 antibody (Transduction Laboratories

Lexington, KY; Fig. 22A) or the rabbit anti-XIAP antibody described above (Fig. 22B).

The anti-CPP32 antibody, which recognizes the CPP32 protease (also known as YAMA or apopain) partitioned almost exclusively in the cytoplasmic fraction. The 55 kDa XIAP protein localized exclusively in the cytoplasm of apoptotic cells, in agreement with the studies presented above, where XIAP protein in normal, healthy COS cells was seen to localize, by immunofluoresence microscopy, to the cytoplasm. In contrast, the 26 kDa cleavage product localized exclusively to the nuclear fraction of apoptotic Jurkat cells. Taken together, these observations suggest that the anti-apoptotic component of XIAP could be the 26 kDa cleavage product, which exerts its influence within the nucleus.

D. In vitro Cleavage of XIAP Protein and Characterization of the Cleavage Product

For this series of experiments, XIAP protein was labeled with ^{35}S using the plasmid pcDNA3-6myc-xiap, T7 RNA polymerase, and a coupled transcription/ translation kit (Promega) according to the manufacturer's instructions. Radioactively labeled XIAP protein was separated from unincorporated methionine by column chromatography using Sephadex G-50™. In addition, extracts of apoptotic Jurkat cells were prepared following treatment with anti-Fas antibody (1 μg/ml) for three hours. To prepare the extracts, the cells were lysed in Triton X-100 buffer (1% Triton X-100, 25 mM Tris HCl) on ice for two hours and then microcentrifuged for 5 minutes. The soluble extract was retained (and was labelled "TX100"). Cells were lysed in cell extraction buffer with freeze/thawing. The soluble cytoplasmic fraction was set aside (and labelled "CEB"). Nuclear pellets from the preparation of the CEB cytoplasmic fraction

were solubilized with Triton X-100 buffer, microcentrifuged, and the soluble fractions, which contains primarily nuclear DNA, was retained (and labelled "CEB-TX100"). Soluble cell extract was prepared by lysing cells with NP-40 buffer, followed by microcentrifugation for 5 minutes (and was labeled NP-40). *In vitro* cleavage was performed by incubating 16 µl of each extract (CEB, TX-100, CEB-TX100, and NP-40) with 4 µl of *in vitro* translated XIAP protein at 37°C for 7 hours. Negative controls, containing only TX100 buffer or CEB buffer were also included. The proteins were separated on a 10% SDS-polyacrylamide gel, which was then dried and exposed to X-ray film overnight.

In vitro cleavage of XIAP was apparent in the CEB extract. The observed molecular weight of the cleavage product was approximately 36 kDa (Fig. 23). The 10 kDa shift in the size of the cleavage product indicates that the observed product is derived from the amino-terminus of the recombinant protein, which contains six copies of the myc epitope (10 kDa). It thus appears that the cleavage product possesses at least two of the BIR domains, and that it is localized to the nucleus.

XIII. Treatment of HIV Infected Individuals

The expression of *hiap-1* and *hiap-2* is decreased significantly in HIV-infected human cells. Furthermore, this decrease precedes apoptosis. Therefore, administration of HIAP-1, HIAP-2, genes encoding these proteins, or compounds that upregulate these genes can be used to prevent T cell attrition in HIV-infected patients. The following assay may also be used to screen for compounds that alter *hiap-1* and *hiap-2* expression, and which also prevent apoptosis.

Cultured mature lymphocyte CD-4$^+$ T cell lines (H9, labelled "a"; CEM/CM-3, labelled "b"; 6T-CEM, labelled "c"; and Jurkat, labelled "d" in Figs. 13A and 13B), were examined for signs of apoptosis (Fig. 13A) and *hiap* gene expression (Fig. 13B) after exposure to mitogens or HIV infection. Apoptosis was demonstrated by the appearance of DNA "laddering" upon gel electrophoresis and gene expression was assessed by PCR. The results obtained from normal (non-infected, non-mitogen stimulated) cells are shown in each lane labelled "1" in Figs. 13A and 13B. The results obtained 24 hours after PHA/PMA (phytohemagglutinin/phorbol ester) stimulation are shown in each lane labelled "2". The results obtained 24 hours after HIV strain III_B infection are shown in each lane labelled "3". The "M" refers to standard DNA markers (the 123 bp ladder in Fig. 13B, and the lambda *Hind*III ladder in Fig. 13A (both from Gibco-BRL)). DNA ladders (Prigent et al., J. Immunol. Meth., 160:139, 1993), which indicate apoptosis, are evident when DNA from the samples described above are electrophoresed on an ethidium bromide-stained agarose gel (Fig. 13A). The sensitivity and degree of apoptosis of the four T cell lines tested varies following mitogen stimulation and HIV infection.

In order to examine *hiap* gene expression, total RNA was prepared from the cultured cells and reverse transcribed using oligo-dT priming. The RT cDNA products were amplified by PCR using specific primers (as shown in Table 5) for the detection of *hiap-2a*, *hiap-2b* and *hiap-1*. The PCR was conducted using a Perkin Elmer 480 thermocycler with 35 cycles of the following program: 94°C for one minute, 55°C for 2 minutes and 72°C for 1.5 minutes. The RT-PCR reaction products were electrophoresed on a 1% agarose gel, which was stained with ethidium bromide. Absence of *hiap-2* transcripts is noted in all four cell lines 24 hours after HIV infection. In three of four cell lines (all except H9), the *hiap-1*

gene is also dramatically down-regulated after HIV infection. PHA/PMA mitogen stimulation also appears to decrease *hiap* gene expression; particularly of *hiap-2* and to a lesser extent, of *hiap-1*. The data from these experiments is summarized in Table 5. The expression of *β-actin* was consistent in all cell lines tested, indicating that there is not a flaw in the RT-PCR assay that could account for the decrease in *hiap* gene expression.

TABLE 4
OLIGONUCLEOTIDE PRIMERS FOR THE SPECIFIC RT-PCR AMPLIFICATION OF UNIQUE IAP GENES

IAP Gene	Forward Primer (nucleotide position*)	Reverse Primer (nucleotide position*)	Size of Product (bp)
h-xiap	p2415 (876-896)	p2449 (1291-1311)	435
m-xiap	p2566 (458-478)	p2490 (994-1013)	555
h-hiap1	p2465 (827-847)	p2464 (1008-1038)	211
m-hiap1	p2687 (747-767)	p2684 (1177-1197)	450
hiap2	p2595 (1562-1585)	p2578 (2339-2363)	801[a] 618[b]
m-hiap2	p2693 (1751-1772)	p2734 (2078-2100)	349

* Nucleotide position as determined from Figs. 1-4 for each IAP gene
[a] PCR product size of *hiap2a*
[b] PCR product size of *hiap2b*

TABLE 5
APOPTOSIS AND HIAP GENE EXPRESSION IN CULTURED T-CELLS FOLLOWING MITOGEN STIMULATION OR HIV INFECTION

Cell Line	Condition	Apoptosis	*hiap1*	*hiap2*
H9	not stimulated	-	+	±
	PHA/PMA stimulated	+++	+	±
	HIV infected	++	+	-
CEM/CM-3	not stimulated	-	+	±
	PHA/PMA stimulated	±	+	-
	HIV infected	±	-	-
6T-CEM	not stimulated	-	+	+
	PHA/PMA stimulated	±	-	-
	HIV infected	+	-	-
Jurkat	not stimulated	-	+	++
	PHA/PMA stimulated	+	+	+
	HIV infected	±	-	-

XIV. Assignment of *xiap*, *hiap-1*, and *hiap-2* To Chromosomes Xq25 and 11q22-23 by Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) was used to identify the chromosomal location of *xiap*, *hiap-1* and *hiap-2*. The probes used were cDNAs cloned in plasmid vectors: the 2.4 kb *xiap* clone included 1493 bp of coding sequence, 34bp of 5' UTR (untranslated region) and 913 bp of 3'UTR; the *hiap-1* cDNA was 3.1 kb long and included 1812 bp coding and 1300 bp of 3' UTR; and the *hiap-2* clone consisted of 1856 bp of coding and 1200 bp of 5' UTR. A total of 1 μg of probe DNA was labelled with biotin by nick translation (BRL). Chromosome spreads prepared from a normal peripheral blood culture were denatured for 2 minutes at 70°C in 50% formamide/2X SSC and subsequently hybridized with the biotin labelled DNA probe for 18 hours at 37°C in a solution consisting of 2X SSC/70% formamide/10% dextran sulfate. After hybridization,

the spreads were washed in 2X SSC/50% formamide, followed by a wash in 2X SSC at 42°C. The biotin labelled DNA was detected by fluorescein isothiocyanate (FITC) conjugated avidin antibodies and anti-avidin antibodies (ONCOR detection kit), according to the manufacturer's instructions. Chromosomes were counterstained with propidium iodide and examined with a Olympus BX60 epifluorescence microscope. For chromosome identification, the slides with recorded labelled metaphase spreads were destained, dehydrated, dried, digested with trypsin for 30 seconds and stained with 4% Giemsa stain for 2 minutes. The chromosome spreads were relocated and the images were compared.

A total of 101 metaphase spreads were examined with the *xiap* probe, as described above. Symmetrical fluorescent signals on either one or both homologs of chromosome Xq25 were observed in 74% of the cells analyzed. Following staining with *hiap-1* and *hiap-2* probes, 56 cells were analyzed and doublet signals in the region 11q22-23 were observed in 83% of cells examined. The *xiap* gene was mapped to Xq25 while the *hiap-1* and *hiap-2* genes were mapped at the border of 11q22 and 11q23 bands.

These experiments confirmed the location of the *xiap* gene on chromosome Xq25. No highly consistent chromosomal abnormalities involving band Xq25 have been reported so far in any malignancies. However, deletions within this region are associated with a number of immune system defects including X-linked lymphoproliferative disease (Wu et al., Genomics 17:163, 1993).

Cytogenetic abnormalities of band 11q23 have been identified in more than 50% of infant leukemias regardless of the phenotype (Martinez-Climet et al., Leukaemia 9:1299, 1995). Rearrangements of the MLL Gene (mixed lineage leukemia or myeloid lymphoid leukemia; Ziemin Van der Poel et al., Proc.

Natl. Acad. Sci. USA 88:10735, 1991) have been detected in 80% of cases with 11q23 translocation, however patients whose rearrangements clearly involved regions other than the MLL gene were also reported (Kobayashi et al., Blood 82:547, 1993). Thus, the IAP genes may follow the Bcl-2 paradigm, and would therefore play an important role in cancer transformation.

XV. Preventive Anti-Apoptotic Therapy

In a patient diagnosed to be heterozygous for an IAP mutation or to be susceptible to IAP mutations (even if those mutations do not yet result in alteration or loss of IAP biological activity), or a patient diagnosed as HIV positive, any of the above therapies may be administered before the occurrence of the disease phenotype. For example, the therapies may be provided to a patient who is HIV positive but does not yet show a diminished T cell count or other overt signs of AIDS. In particular, compounds shown to increase IAP expression or IAP biological activity may be administered by any standard dosage and route of administration (see above). Alternatively, gene therapy using an IAP expression construct may be undertaken to reverse or prevent the cell defect prior to the development of the degenerative disease.

The methods of the instant invention may be used to reduce or diagnose the disorders described herein in any mammal, for example, humans, domestic pets, or livestock. Where a non-human mammal is treated or diagnosed, the IAP polypeptide, nucleic acid, or antibody employed is preferably specific for that species.

Other Embodiments

In other embodiments, the invention includes any protein which is substantially identical to a mammalian IAP polypeptides (Figs. 1-6; SEQ ID NOs: 1-42); such homologs include other substantially pure naturally-occurring mammalian IAP proteins as well as allelic variants; natural mutants; induced mutants; DNA sequences which encode proteins and also hybridize to the IAP DNA sequences of Figs. 1-6 (SEQ ID NOS: 1-42) under high stringency conditions or, less preferably, under low stringency conditions (e.g., washing at 2X SSC at 40^0C with a probe length of at least 40 nucleotides); and proteins specifically bound by antisera directed to a IAP polypeptide. The term also includes chimeric polypeptides that include a IAP portion.

The invention further includes analogs of any naturally-occurring IAP polypeptide. Analogs can differ from the naturally-occurring IAP protein by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the invention will generally exhibit at least 85%, more preferably 90%, and most preferably 95% or even 99% identity with all or part of a naturally occurring IAP amino acid sequence. The length of sequence comparison is at least 15 amino acid residues, preferably at least 25 amino acid residues, and more preferably more than 35 amino acid residues. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring IAP polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as

described in Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual* (2d ed.), CSH Press, 1989, or Ausubel et al., *supra*). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or nonnaturally occurring or synthetic amino acids, e.g., β or γ amino acids. In addition to full-length polypeptides, the invention also includes IAP polypeptide fragments. As used herein, the term "fragment," means at least 20 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of IAP polypeptides can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

Preferable fragments or analogs according to the invention are those which facilitate specific detection of a IAP nucleic acid or amino acid sequence in a sample to be diagnosed. Particularly useful IAP fragments for this purpose include, without limitation, the amino acid fragments shown in Table 2.

What is claimed is: